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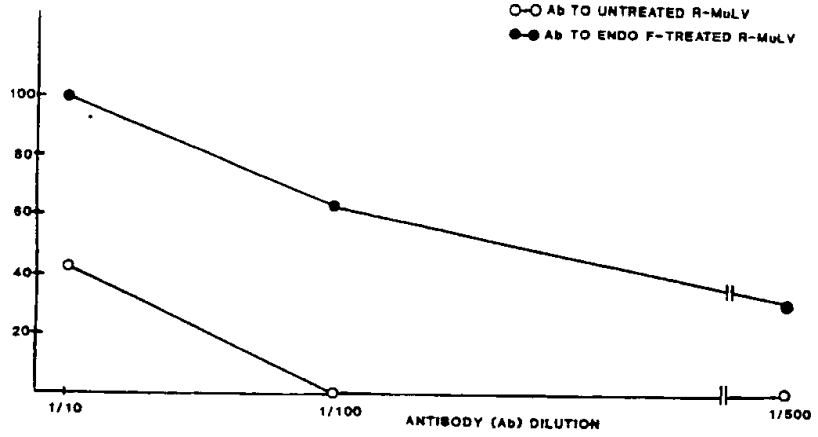
(54) Title: POLYPEPTIDES AND ANTIBODIES RELATED TO DEGLYCOSYLATED VIRAL GLYCOPROTEINS



(57) Abstract

An N-linked, carbohydrate-free polypeptide comprising an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, its related nor-virus whose envelope protein is free of N-linked carbohydrate, and methods of producing and utilizing the polypeptide. The polypeptide is substantially free of N-linked glycosylation of the glycoprotein and induces the production of antibody-containing serum that (i) provides improved cross-reactivity among related viral strains and/or (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by the viral envelope N-linked glycoprotein when the polypeptide and the viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

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POLYPEPTIDES AND ANTIBODIES RELATED TO DEGLYCOSYLATED VIRAL GLYCOPROTEINS

DESCRIPTION

Technical Field

The present invention relates to polypeptides comprising an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, and more particularly to polypeptides and nor-viruses that are substantially free of the N-linked glycosylation of a viral envelope N-linked glycoprotein and are capable of inducing the production of antibody-containing serum that (i) provides improved cross-reactivity among related viral strains and/or (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by the viral envelope N-linked glycoprotein.

Background of the Invention

The pathways and mechanisms by which carbohydrate moieties (glycosyl groups) are added to glycoproteins are now relatively well defined, Hubbard et al., Ann. Rev. Biochem., 50, 555 (1981). However, heretofore little has been known about the role carbohydrates play in the function of this class of proteins.

Possible roles posed for glycosylation include increasing hydrophilicity of certain regions of a given molecule, protection of the molecule from proteolytic attack, facilitation of mobilization to the cell surface and dictation of secretion of certain proteins. See, for example, Gibson et al., Trends Biochem. Sci., 5, 290 (1980); Schwarz et al., Trends Biochem. Sci., 5, 65 (1980); Heifetz et al., Biochemistry, 18, 2186 (1979).

Although examples of each of the above suggestions are evident, generalizations regarding the role of the carbohydrate on glycoproteins as a class are not apparent. See, Sharon et al., The Proteins, Volume V, 3d ed., Neurath et al., eds., Academic Press, New York (1982).

There has been much speculation in the literature regarding the role(s) carbohydrate may play in a host animal's immune response to viruses, in particular for retroviruses and influenza viruses. See, Wunner et al., J. Gen. Virol., 64, 1649 (1983); and Snyder et al., Proc. Natl. Acad. Sci, (USA), 77, 1622 (1980).

Several studies have been reported wherein bovine leukemia virus glycoproteins were treated with glycosidases, exoglycosidases, trypsin, and/or proteases, and then examined for antigenic activity. In each case, it was reported that the carbohydrate moiety of the glycoprotein was essential for stimulation of effective immunogenic activity against the virus in cattle. Bruck et al., Virology, 136, 20 (1984); Miller et al., "Vaccination with Glycosidase-Treated Glycoprotein Antigen Does Not Prevent Bovine Leukemia Virus Infection in Cattle", 5th International Symposium of Bovine Leukosis, Tubingen, October 19-21, 1982; Schwerr et al., Virology, 109, 431 (1981); and Portetelle et al., Virology, 105, 223 (1980).

Thus, in order to use a glycoprotein as an immunogen, it has heretofore been thought that the presence of carbohydrate groups was critical for the glycoprotein to elicit an effective immune response. One reason for that conclusion may be that glycosidase preparations have typically been

contaminated with proteases that cleave the glycoproteins into non-immunogenic fragments.

It has also been reported that when the purified glycoprotein called gp71 of Friend murine leukemia virus was treated with endoglycosidase, 70 percent of the carbohydrate was removed without affecting type, group, or interspecies antigenic reaction as detectable in radioimmunoassays or adsorption of cytotoxic antibodies, Schafer et al., J. Virology, 21, 35 (1977); and Bolognesi et al., J. Virology, 16, 1453 (1975). However, while antigenicity was examined for the deglycosylated glycoprotein, the immunogenic effects of the deglycosylated protein were not considered.

The immunogenic contributions of the carbohydrate and polypeptide portions of the major envelope glycoprotein (gp90) of the equine infectious anemia virus called EIAV have been analyzed by Montelaro et al., Virology, 136, 368 (1984). The analysis was made by measuring the effects of specific glycosidase and protease digestions on the reactivity of the glycoprotein with immune sera from infected horses. The results of both direct and competitive radioimmunoassays indicated that immune sera contained antibodies reactive with both carbohydrate and protein moieties of EIAV gp90, with the predominant reactivity apparently being against the gp90 polypeptide epitopes.

Those results may be contrasted with descriptions of retrovirus glycoprotein antigenicity in which antigenic reactivity was attributed exclusively to protein, Bolognesi et al., J. Virology, supra; and Van Eldrik et al., Virology, 86, 193 (1978), or to carbohydrate, Schmerr et al., Virology, supra; and Portetelle et al., Virology,

supra. See also, Ho-Terry et al., Arch. Virology, 79, 139 (1984); and Salabe et al., Clin. Exp. Immunol., 25, 234 (1976). However, the usefulness of glycosidase-treated glycoprotein as an immunogen was again not analyzed in any of the above reports.

The loss of tumor transplantation of L1210 leukemic tumor cells after treatment with neuraminidase, that liberates sialic (N-acetylneuraminic) acid moieties by cleaving their O-glycoside links with underlying amino-sugars, has been reported by Bagshawe et al., Nature (London), 218, 1254 (1968). There, mice were inoculated with 40,000 L1210 tumor cells, receiving all cells treated with neuraminidase or all untreated cells. All mice that received 40,000 untreated tumor cells died within 20 days, but mice that received tumor cells treated with neuraminidase remained in apparent normal health for more than 100 days. However, neither the use of the deglycosylated tumor cells as an immunogen nor the use of deglycosylated viruses was disclosed.

Summary of the Invention

The present invention contemplates a polypeptide free of N-linked carbohydrate having an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, as well as methods of producing and utilizing the polypeptide.

In one aspect of the invention, a polypeptide having an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein is contemplated. The polypeptide is substantially free of the N-linked glycosylation of the glycoprotein and induces the

production of antibody-containing serum that (i) exhibits improved cross-reactivity among related viral strains and/or (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by the viral envelope N-linked glycoprotein when the polypeptide and the viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

Another aspect of the present invention contemplates a modified virus or virion described herein as a nor-virus or nor-virion whose envelope protein has an amino acid residue sequence substantially identical to that of the unmodified virus envelope protein, the latter envelope protein containing N-linked glycosylation in the unmodified state. The envelope protein of the nor-virus is substantially free of carbohydrate moieties covalently linked to asparagine residues (N-links) and induces the production of antibody-containing serum that (i) exhibits improved cross-reactivity among related viral strains and/or (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by the virus when the polypeptide and the virus are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

In yet another aspect of the present invention, an inoculum against infection by a retrovirus or a myxovirus is contemplated. The

inoculum includes an effective amount of the above-described polypeptide or nor-virus of the invention in a physiologically tolerable diluent. The inoculum, when introduced into a host, is capable of inducing the production of antibodies in the host that immunoreact with the nor-virus, the virus, and with a related viral strain, and neutralize the virus in vitro. Preferably, such inocula are utilized to protect the host from in vivo viral infection.

In a further aspect of the present invention, antibodies to a retrovirus or a myxovirus are contemplated. The antibodies are raised in an animal host to the above-described polypeptide or nor-virus of the invention and have the capacity to immunoreact with the virus, the nor-virus, and a related viral strain as well as neutralize the virus in vitro.

In yet another aspect of the present invention, a method for the production of antibodies to a virus is contemplated. The method comprises (i) introducing into an animal host an effective amount of the above-described polypeptide or nor-virus of the invention that is capable of inducing the production of antibodies in the serum of the host; and (ii) collecting induced antibody-containing serum from the host. The antibodies may be recovered in purified form from the induced antibody-containing serum by well known techniques, such as affinity purification. The antibodies and antibody-containing serum so prepared immunoreact with the virus, the nor-virus, a related viral strain and also neutralize the virus in vitro.

In a still further aspect of the present invention, a method of improving the immunogenicity of a viral envelope N-linked glycoprotein is

contemplated. The method comprises (i) providing a viral N-linked envelope glycoprotein or virus having an N-linked envelope glycoprotein; (ii) reacting the glycoprotein with a glycosidase such as an endoglycosidase or exoglycosidase to remove glycosyl groups from the glycoprotein to form the above-described polypeptide or virus, respectively. The deglycosylated polypeptide or virus (the nor-virus) so formed, when utilized in an effective amount in an inoculum, is capable of inducing the production of antibody-containing serum that (i) exhibits improved cross-reactivity among related viral strains and/or (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by the corresponding glycoprotein or virus, respectively, when the polypeptide or nor-virus and the respective glycoprotein or virus are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

Still another aspect of the present invention, contemplates a method of immunizing an animal against a virus. The method comprises (i) providing a unit dose of inoculum comprising the above-describe polypeptide or nor-virus of the present invention dispersed in an effective amount in a physiologically tolerable diluent, the polypeptide or nor-virus having the capacity to induce the production of antibodies in the animal that immunoreact with the virus, nor-virus, a related viral strain as well as neutralize the virus <u>in vitro</u>, and preferably protect the animal from the virus; and (ii) introducing the unit dose of the

inoculum into the blood stream of the animal to be immunized.

The present invention provides several benefits and advantages.

One benefit of the present invention is that the polypeptide or nor-virus of the invention provides improved immune system susceptibility to viral immunogens.

One of the advantages of the present invention is that antibodies raised against the polypeptide or nor-virus of the invention display a greater neutralizing titer than do antibodies raised against a corresponding glycoprotein or a fully glycosylated virus, respectively, when used as immunogens in separate animals of the same strain under similar immunization conditions, and thus provide an improved method of immunizing animals against viruses.

A further advantage of the present invention is that antibodies raised against the polypeptide or nor-virus of the invention provide improved cross-reactivity among related viral strains, thereby providing the ability to use a nor-virus or carbohydrate-free viral envelope polypeptide of this invention in an inoculum against another, related virus.

Other advantages and benefits of the present invention will become readily apparent to those skilled in the art from the following description of the invention, the drawings and the appended claims.

Brief Description of the Drawings

In drawings forming a portion of the disclosure of this invention:

FIGURE 1 is a photograph of a Western blot analysis visualized by autoradiography showing the reactivity of heteroantisera to Rauscher murine leukemia virus (R-MuLV) glycoprotein gp70 before and after deglycosylation with Endoglycosidase F (Endo F), Elder and Alexander, Proc. Natl. Acad. Sci. (USA), 79, 4540 (1982). Immune reactivity was assessed against R-MuLV either untreated (native) or deglycosylated with Endo F to form a nor-virus as described in Elder and Alexander, supra, followed by separation on SDS-PAGE, as in Laemmli, Nature, 227, 680 (1970), and transfer to nitrocellulose [Western blotting as described in Towbin et al., Proc. Natl. Acad. Sci. (USA), 76, 4350 (1979) and modified in Johnson et al., Gene Anal. Techn., 1, 3 (1984)].

Lane A shows reactivity of R-MuLV gp70 to a goat heteroserum prepared against purified R-MuLV gp70, as described in Elder et al., Nature, 267, 23 (1977), before (-) and after (+) treatment with Endo F. Lane B shows reactivity of goat heteroserum made against intact virus (sera obtained from the Resources Branch of the National Cancer Institute) with R-MuLV proteins before (-) and after (+) treatment of those proteins with Endo F. Lane C shows reactivity of a goat heteroserum made against TWEEN-ether disrupted virus (sera obtained from the Resources Branch of the National Cancer Institute) before (-) and after (+) treatment with Endo F. As used herein, the term "TWEEN" is used to indicate polyoxyethylene (20) sorbitan monooleate. Arrows denote bands corresponding to gp70 before and after Endo F treatment, as is appropriate for the lanes.

Radioactive bands were removed and counted to quantitate changes in reactivity following deglycosylation. Reaction with a monoclonal antibody

that recognized both untreated and deglycosylated gp70 verified that the relative amounts of gp70 remained unchanged after deglycosylation.

FIGURE 2 is a photograph of a Western blot analysis visualized by autoradiography showing the effect of deglycosylation of Rauscher murine leukemia virus (R-MuLV) glycoprotein gp70 on reactivity with monoclonal antibodies. Immunoprecipitations were performed using a panel of 78 monoclonal antibodies made against purified R-MuLV gp70, as described in Niman and Elder, Virology, 123, 189 (1982). R-MuLV disrupted with Nonidet P-40 [polyoxyethylene (9) octyl phenyl ether; Sigma Chemical Co., St. Louis, MO] and radiolabeled with ^{125}I (Amersham, Arlington Heights, IL) was used as antigen. Samples were deglycosylated by treatment with Endo F that removed substantially all N-linked glycans from the glycoproteins, Elder and Alexander, Proc. Natl. Acad. Sci. (USA), supra. The precipitates were then separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and visualized by autoradiography, as described before. Reactivities before (-) and after (+) deglycosylation are shown.

A complete variety of responses was obtained. Twenty-six of the antibodies gave only background bands in immune precipitation (Lane A); six reacted only after carbohydrate removal (Lane B); eight monoclonals reacted only with the untreated gp70 (Lane C); twenty-nine improved in reactivity to various degrees following deglycosylation (Lanes D-E); and nine reacted equally well before and after deglycosylation (Lane F).

FIGURE 3 is a photograph of a Western blot analysis visualized using peroxidase-coupled goat anti-rabbit IgG, hydrogen peroxide and o-dianisidine as colorant showing the reactivity of anti-

synthetic peptide antisera to the X47 influenza hemagglutinin (HA) before and after deglycosylation with Endo F.

X47 influenza virus (H_3N_2 subtype) was grown in embryonated eggs. The virus was concentrated from allantoic fluid by centrifugation.

One-half of the viral sample was treated with Endo F, while the other portion was used as a buffer control. Samples of the glycosylated and deglycosylated virus representing approximately 10^6 plaque forming units of virus were run on adjacent lanes by SDS-PAGE, Laemmli, Nature, supra, and subsequently blotted onto nitrocellulose, Towbin et al., Proc. Natl. Acad. Sci. (USA), supra, and Johnson et al., Gene Anal. Techn., supra. Strips containing a lane of each of the glycosylated and deglycosylated samples were incubated with the various rabbit anti-synthetic peptide antisera made against synthetic peptides corresponding to regions of the X47 influenza HA molecule, as in Green et al., Cell, 28, 477 (1982) and Min Jou et al., Cell, 19, 683 (1980). After washing, the blots were incubated with peroxidase-coupled goat anti-rabbit IgG. The blots were developed with hydrogen peroxide and o-dianisidine. Reactivities before (-) and after (+) deglycosylation with Endo F are shown.

Lane A shows that reactivity of rabbit heteroserum against intact X47 influenza virus decreases dramatically with the deglycosylated HA molecule. Lane B shows that anti-synthetic peptide serum 15 (HA sequence positions 140-156 that contain no site of glycosylation) reacts with neither glycosylated nor deglycosylated HA. Lane C shows that anti-synthetic peptide serum 2 (HA sequence positions 1-36 that contain 2 sites of glycosylation

at positions 8 and 23) improves in reactivity when the HA is deglycosylated. Lane D shows that anti-synthetic peptide 17 (HA sequence positions 174-196 with no site of glycosylation) reacts equally well with either glycosylated or deglycosylated HA. The arrows denote bands corresponding to the influenza HA molecule before and after Endo F treatment, as is appropriate.

FIGURE 4 is a graph showing the adsorption of neutralizing antibodies by deglycosylated Rauscher murine leukemia virus (R-MuLV). The ordinate shows the percent of neutralization and the abscissa shows antibody dilution over an average of five adsorption studies. Antibodies raised to non-deglycosylated R-MuLV (Δ-Δ) were used as a control and adsorption of neutralizing antibodies by untreated R-MuLV (O-O) and by R-MuLV deglycosylated with Endo F (●-●) were compared.

FIGURE 5 is a photograph of a Western blot analysis visualized using peroxidase-coupled goat anti-rabbit antibodies, hydrogen peroxide and 4-chloro-2-naphthol as described in Section III C, and shows the reactivity of antisera raised against R-MuLV or deglycosylated, nor-R-MuLV. The R-MuLV gp70 was deglycosylated (treated) with Endo F thereby providing the deglycosylated protein called as p49. The left panel of lanes shows the reactivity of antisera raised against deglycosylated R-MuLV to native and deglycosylated R-MuLV. The right panel of lanes shows the reactivity of antisera raised against native R-MuLV to native and deglycosylated R-MuLV.

FIGURE 6 is a graph showing the neutralization of R-MuLV by antisera raised against a control and against deglycosylated R-MuLV. The ordinate shows the percent of neutralization and the abscissa shows the antibody dilution. The

neutralization of untreated R-MuLV (O-O) was compared to that of R-MuLV treated with Endo F (nor-R-MuLV) (●-●).

FIGURE 7 is a graph showing the neutralization of feline leukemia virus (FeLV) by antisera raised against untreated FeLV (O-O) and deglycosylated FeLV (nor-FeLV) (●-●). As above for FIGURE 6, the ordinate shows the percent of neutralization and the abscissa shows the antibody dilution.

FIGURE 8 is a photograph of a Western blot analysis visualized as described in FIGURE 5 showing the relative lack of cross-reactivity of antisera raised against native FeLV with R-MuLV. The left lane shows the reactivity of the antisera to native and deglycosylated R-MuLV. The right lane shows the reactivity of the antisera to native and deglycosylated FeLV.

FIGURE 9 is a photograph of a Western blot analysis visualized as described in FIGURE 5 showing cross-reactivity of antisera raised against deglycosylated, nor-FeLV. The left lane shows the reactivity of the antisera to native and to deglycosylated R-MuLV. The right lane shows the reactivity of the antisera to native and to deglycosylated, nor-FeLV.

Detailed Description of the Invention

The present invention is directed to a polypeptide that is free of N-linked carbohydrate that has an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, its related nor-virus whose envelope protein is free of N-linked carbohydrate, and to methods of producing and utilizing same. The polypeptide is substantially free of glycosylation of

the N-linked glycoprotein and induces the production of antibody-containing serum that (i) provides improved cross-reactivity among related viral strains and/or (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by the viral envelope N-linked glycoprotein when the polypeptide and the viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

A polypeptide of this invention is a nor- or deglycosylated form of a native viral envelope glycoprotein that contains a plurality of asparagine-linked (N-linked) carbohydrate moieties in the native form. Such a polypeptide is substantially the same length and has substantially the identical amino acid residue sequence as does the native envelope glycoprotein.

An alternative description of such a polypeptide is that it is a deglycosylation reaction product of an N-linked viral envelope glycoprotein. Particularly preferred polypeptides are reaction products of an endoglycosidase or exoglycosidase, such as neuraminidase, and an N-linked viral envelope glycoprotein. In most preferred practice, the endoglycosidase is the enzyme referred to herein as Endo F and the viral envelope glycoprotein is that of a retrovirus such as a leukemia-inducing retrovirus or a myxovirus such as an influenza virus. In all instances, the polypeptide reaction product has substantially the same amino acid residue length and substantially identical amino acid residue sequence as does the native viral envelope glycoprotein.

By referring to the polypeptide as having substantially the same length as the native glycoprotein, it is meant that the polypeptide may differ by a few residues, e.g., about 5 to about 20 residues, from the native glycoprotein. However, as compared to the native glycoprotein, the polypeptide is relatively free from proteolytic cleavage that reduces its amino acid residue sequence to less than about 90 percent, or more preferably, less than about 95 percent, of the number of residues present in the native glycoprotein. Most preferably, the polypeptide has the same number of amino acid residues as does the native glycoprotein. Thus, deglycosylation of the N-linked carbohydrate moieties does not substantially reduce the length of the native viral envelope protein.

Similarly, by referring to the polypeptide as having a substantially identical amino acid residue sequence to that of the native glycoprotein, it is meant that the amino acid residues present in the polypeptide are also present in the glycoprotein. Thus, for example, if a few amino acid residues have been lost by proteolysis during the deglycosylation reaction, those remaining in the polypeptide are also present in the glycoprotein.

A free, uncombined polypeptide molecule of this invention is most preferably prepared from an N-linked viral envelope glycoprotein of a native virus as contrasted to the possible preparation by recombinant DNA technology of a non-glycosylated or glycosylated protein of the same amino acid residue sequence. It is believed that the three-dimensional structure imposed upon the envelope protein during its synthesis and glycosylation is of some importance to the preparation and presentation of naturally

occurring epitopes that induce production of neutralizing and/or protective antibodies.

The term polypeptide used in relation to a before-described product is meant to encompass a reaction product as a free molecule as well as a reaction product molecule in combined form such as the envelope protein of an intact virus or partially disrupted virus. Thus, for example, an intact virus having an envelope glycoprotein containing N-linked carbohydrate moieties may be deglycosylated by reaction with a deglycosylase to form a nor-virus that previously contained N-linked carbohydrate moieties and is substantially free of N-linked carbohydrate moieties. Since such a nor-virus includes the before-described polypeptide in a combined form as the viral envelope "protein", that nor-virus is considered herein to be within those materials encompassed by the before-described term "polypeptide". Contrarily, the term "nor-virus" is used more narrowly herein, and excludes the before-described polypeptide as a free molecule. When the phrase "polypeptide or nor-virus" appears herein, it is meant to encompass the free, uncombined polypeptide molecule and the combined viral or disrupted viral form of that molecule, respectively.

Definitions

The following definitions are provided to assist the reader in understanding the following disclosure and discussion.

Virus or virion -- An infectious agent that lacks independent metabolism and is able to replicate only within a living host cell. The individual virus particle (virion) consists of DNA or RNA and a protein shell or envelope.

Nor-virus or nor-virion -- A virus particle or virion that normally contains asparagine-linked (N-linked) carbohydrate moieties from which such N-linked carbohydrates have been removed.

Native -- The word "native" used with respect to a virus or protein refers to the usual chemical state of that virus or protein even though the virus or protein may be made by man's intervention through mutation. Thus, a native virus or protein as used herein contains N-linked carbohydrate moieties.

Heteroserum or Heteroantiserum -- Antibody-containing serum that contains antibodies that immunoreact with a plurality of epitopes from a plurality of antigenic determinants at several places on the immunogenic entity, e.g., virus, protein, or glycoprotein, when that entity is used as an antigen.

Oligoclonal serum -- Antibody-containing serum that contains antibodies that immunoreact with one or more epitopes of a synthetic peptide immunogen that corresponds to an antigenic determinant of a viral, protein or glycoprotein immunogen. Oligoclonal sera typically have a narrower specificity than do hetersera.

Monoclonal antibodies -- Antibodies or serum-containing antibodies having specificity for a single epitope of a single antigenic determinant of the immunogenic entity. Monoclonal antibodies may be contaminated with minor amounts of antibodies that are typically unrelated to the immunogenic entity and are typically artifacts of preparation, as where ascites is used.

Sequence substantially identical -- The term "sequence substantially idential" is used herein to mean little if any cleavage of proteins, particularly

in the middle of the sequence, although a few amide bonds at the ends of the protein may be cleaved.

I. General Discussion

The ability of heteroantisera, monoclonal antibodies and anti-synthetic peptide (oligoclonal) antibodies to immunoreact with deglycosylated viral glycoproteins has been examined. The results discussed hereinafter indicate that the reactivities of the majority of antibodies (antibody-containing sera) raised against these glycoproteins were markedly influenced by the attached carbohydrate (glycosyl) moieties.

All heteroantisera prepared in rabbits or goats to either fully glycosylated retrovirus or influenza virus were virtually unreactive with the deglycosylated viral envelope glycoproteins after carbohydrate removal. Assays using a selected panel of monoclonal antibodies raised against purified Rauscher murine leukemia virus (R-MuLV) envelope glycoprotein gp70 indicated that a large number (35 of 78) of those antibodies exhibited improved reactivity, while the immunoreactivity of other monoclonal antibodies decreased or remained unchanged (17 of 78) after carbohydrate was removed from the gp70 antigen. The remaining monoclonal antibodies (26 of 78) showed only background reactivities. The majority of anti-synthetic peptide (oligoclonal) antibodies to influenza virus hemagglutinin also exhibited improved immunoreactivity after carbohydrate was removed from the hemagglutinin antigen molecule.

These assays demonstrated that (1) carbohydrate side chains on viral glycoproteins influence the immune response to these antigens; and (2) the more native the glycoprotein immunogen, the

more prominent the carbohydrate influence observed. The results discussed hereinafter also indicate that the immune response to the glycoproteins is not simply a function of the immunogenicity of certain domains over others, but is rather a direct measure of carbohydrate influences on the host animal's immunological response to the foreign immunogen (antigen).

The assays discussed were facilitated by the use of Endoglycosidase F, Elder and Alexander, Proc. Natl. Acad. Sci. (USA), supra, that efficiently cleaves both N-linked high mannose and complex glycans from glycoproteins, and thus permits direct screening of aspects of the carbohydrate-protein interaction. The polypeptides of the invention were obtained from viral envelope glycoproteins, illustratively, by immunoreaction with Endo F. However, they may also be obtained by immunoreaction with endoglycosidases called Endo H and Endo D, Elder and Alexander, supra, or by immunoreaction with neuraminidase, an exoglycosidase. The deglycosylation of R-MuLV envelope glycoprotein gp70 results in the formation of a protein called p49.

Further assays were undertaken to characterize the relationship between those antibodies influenced by carbohydrate and the antibodies responsible for virus neutralization. The results discussed hereinafter illustrate that carbohydrate directs the immune response to determinants other than those involved in viral infectivity. Surprisingly, it was found that viral antigens exhibited a relatively stronger neutralizing response after carbohydrate removal. This result was directly opposite to those previously reported, Bruck et al., Virology, supra; Miller et al., 5th

International Symposium of Bovine Leukosis, supra; Schmerr et al., Virology, supra; Portetelle et al., Virology, supra.

The initial assay in this regard was performed based upon the observation that all heterosera or oligoclonal sera raised against retroviruses (murine, feline and primate) as well as influenza virus were virtually unreactive to their respective envelope glycoprotein antigens by immune precipitation or Western blotting after carbohydrate was removed from these antigens by treatment with Endo F. As the surface glycoproteins are the primary targets of virus neutralization, it was expected that neutralization of deglycosylated virus would be similarly diminished.

Since complete deglycosylation by Endo F involves relatively harsh treatment that can diminish viral infectivity in itself, assays were performed using native (envelope glycoprotein) and deglycosylated (nor-glycoprotein) antigens to adsorb antisera, followed by evaluation of the neutralization titers of the sera against fully infectious viruses. Surprisingly, it was found that deglycosylated Rauscher murine leukemia virus (nor-R-MuLV) was able to adsorb neutralizing antibodies as well as, if not better than, native viral antigen. Thus, the results discussed hereinafter illustrate that N-linked envelope carbohydrates dramatically affect the overall immune response to the viral glycoproteins, but to regions not involved in virus infectivity.

It was also desired to determine what influence carbohydrate removal has on the efficacy of the virus as an immunogen. Two aliquots of Rauscher murine leukemia virus (R-MuLV) were prepared

identically, except that one sample was treated with Endo F to deglycosylate the surface glycoprotein, gp70 to form p49, while the other aliquot was left in its native form.

These immunogens were dispersed in physiologically tolerable diluents and were subsequently introduced by injection in rabbits. The resultantly raised (induced) antisera were examined in both Western blotting and neutralization assays.

As expected, untreated, native R-MuLV elicited antibodies that only reacted by Western blotting with non-deglycosylated, native gp70. Deglycosylated R-MuLV (nor-R-MuLV), however, induced antibodies that reacted equally well with the native envelope glycoprotein, gp70, and with the nor-glycoprotein, p49. This was an unexpected result as it would be expected that antibodies raised against deglycosylated immunogens would fail to react or would exhibit diminished reactivity against the native envelope glycoprotein antigen. Furthermore, the overall antibody response to all the viral proteins was markedly enhanced by removal of carbohydrate from the gp70.

The results discussed below were obtained using polypeptides of the invention comprising amino acid residue sequences substantially identical to those of Rauscher murine leukemia virus, (R-MuLV), feline leukemia virus (FeLV) and influenza virus envelope N-linked glycoproteins. However, polypeptides of the invention also include polypeptides comprising amino acid residue sequences substantially identical to those of any virus generally, and particularly to those of leukemia virus and myxovirus envelope N-linked glycoproteins.

Leukemia viruses (retroviruses) include a group of RNA viruses causing leukemia and/or leucocyte-related tumors in animals. These viruses include avian leukosis virus, Rous sarcoma and murine leukemia viruses, Friend leukemia virus, Rauscher murine leukemia virus and feline leukemia virus, those human leukemia viruses called HTLV I, HTLV II and HTLV III, as well as Mink Cell Focus-Forming Viruses (MCF) and Adult T-Cell Leukemia Virus (ATLV). Myxoviruses include a group of viruses, such as the viruses of influenza, parainfluenza, mumps and Newcastle disease, that characteristically cause agglutination of erythrocytes.

Therefore, it is to be understood that the results discussed hereinbelow are illustrative of embodiments utilizing polypeptides substantially identical in sequence to Rauscher murine leukemia virus, feline leukemia virus and influenza virus envelope N-linked glycoproteins, but the present invention is not intended to be so limited.

II. Results

A. Influence of Glycosylation on Immune Reactivity of Antisera Prepared Against Viral Glycoproteins

The abilities of heteroantisera and monoclonal antibodies to react with viral glycoproteins deglycosylated with Endo F were assayed using Rauscher murine leukemia virus (R-MuLV). FIGURE 1 shows the results of Western blot analyses of untreated (native) and deglycosylated R-MuLV using heterosera prepared to purified R-MuLV glycoprotein (gp70), intact R-MuLV, and TWEEN-ether disrupted R-MuLV.

All three antisera showed diminished reactivity to gp70 after treatment of the virus with Endo F. The heteroserum prepared against purified gp70 immunoreacted to only 40 of percent control levels after carbohydrate removal (calculated by removing and counting the radioactive bands from nitrocellulose strips) as shown in FIGURE 1A.

Antiserum raised to whole, native unmodified virus, whether prepared against intact (FIGURE 1B) or TWEEN-ether disrupted (FIGURE 1C) viruses, reacted with deglycosylated gp70 at the level of 1-3 percent of the reactivity observed against untreated, native virus. Immunoreactivity of the anti-whole virus antisera to the nonglycosylated core protein p30 (major band at a molecular weight of 30,000 daltons in FIGURES 1B-C) remained unchanged after Endo F treatment, underscoring the glycoprotein specificity of the response. These results indicate that the presence of carbohydrate greatly influenced the immune response when the glycoprotein immunogen was presented in a relatively native conformation (i.e., presented as whole virus).

In FIGURE 2, representative immune precipitations using anti-R-MuLV gp70 monoclonal antibodies, as in Niman and Elder, Virology, supra, versus untreated and deglycosylated R-MuLV are shown. The results of this assay demonstrate that some monoclonal antibodies immunoprecipitated control and Endo-F treated gp70 very poorly (FIGURE 2A), others precipitated gp70 only after carbohydrate was removed (FIGURE 2B), and another group only reacted with untreated R-MuLV gp70 (FIGURE 2C).

The majority of these antibodies (35 of 52 immune precipitating monoclonal antibodies) improved in reactivity to various degrees following

deglycosylation of gp70 (FIGURES 2D-E). Still others reacted equally well with both treated and untreated gp70 (FIGURE 2F).

The above results demonstrate that the majority of the above panel of monoclonal antibodies were markedly influenced by carbohydrate moieties on R-MuLV gp70. Most of this influence was not via actual contribution of glycan to the epitope recognized by the antibody, since the majority of the monoclonal antibodies improved in reactivity after carbohydrate removal. A small proportion (8 of 52 immune precipitating antibodies) failed to react with R-MuLV gp70 after glycans were removed and thus may have recognized carbohydrate as all or part of an epitope. However, the carbohydrate removal could have caused a shift in the conformation of the polypeptide backbone such that the epitope was no longer recognized.

The effects of glycosylation on the immune response to glycoproteins were further assayed by using the X47 influenza virus (H_3N_2 subtype) hemagglutinin (HA) molecule, a molecule for which the entire primary structure, including glycosylation sites, was known, Min Jou et al., Cell, supra, and for which anti-synthetic peptide antisera directed against peptides spanning greater than 75 percent of the entire HA molecule were available, Green et al., Cell, 28, 477 (1982). Representative results obtained by Western blotting are shown in FIGURE 3.

When a rabbit heteroantiserum made against X47 influenza virus was immunoreacted with glycosylated virus and deglycosylated virus (nor-virus) (FIGURE 3A), results strikingly similar to those obtained with heteroantisera against the retroviruses were obtained (FIGURE 2B); namely,

deglycosylation substantially reduced to abolished the reactivity of the anti-influenza virus antiserum.

Five of the twenty anti-synthetic peptide antibodies gave no reaction either with or without deglycosylation, similar to results with normal rabbit serum (FIGURE 3B). Of the ten oligoclonal sera made against defined regions of the influenza HA molecule that contained attached carbohydrate moieties, seven showed improved reactivity (FIGURE 3C) with the deglycosylated molecule, one had unchanged reactivity, and two were unreactive with either the glycosylated or deglycosylated form of the HA molecule.

In addition, ten anti-synthetic peptide antibodies directed against influenza HA sequences were screened that did not contain sites of glycosylation. Of these, three antisera improved in reactivity following deglycosylation of the HA molecule, four antisera were unchanged in reactivity (FIGURE 3D) and three showed no reactivity. Thus, the majority of site-specific antibodies directed at a sequence of the influenza HA molecule containing a site of glycosylation improved in reactivity against the HA molecule after removal of its carbohydrate moiety.

The above results illustrate the direct involvement of the carbohydrate moieties of glycoproteins with the host's immune recognition and response to these molecules. The assays using monoclonal antibodies to the R-MuLV gp70 molecule and the oligoclonal antibodies specifically directed to regions of the influenza HA molecule (containing sites of carbohydrate attachment) demonstrated that the carbohydrate portion of these glycoproteins can

effectively block the interaction of antibodies with the underlying polypeptide regions.

In the influenza system this was true even with regions of the HA molecule that did not have attached carbohydrate, presumably by some distal effect. By way of contrast, heteroantisera made to either R-MuLV or influenza virus lost virtually all reactivity toward the gp70 and HA molecules, respectively, after deglycosylation. This latter result shows that not only can the carbohydrate moieties block the interaction of antibodies to protein antigenic determinants, but that the carbohydrate either becomes the major immunogenic target of the glycosylated protein or directs the immune response to areas under influences of carbohydrate attachment. Thus, the carbohydrate moieties perform a dual role by masking certain polypeptide sites and by simultaneously directing the immune response so as to act as immune decoys.

B. Neutralization Assays

Assays were conducted to determine whether deglycosylated R-MuLV could adsorb out the neutralizing antibodies from heterosera that failed (or were weak) to react by Western blotting with deglycosylated surface glycoprotein gp70. To do so, a quantity of R-MuLV were inactivated and then split into two aliquots, one of which was treated with Endo F to remove carbohydrates to form nor-viruses containing p49 as the envelope "protein". The two resulting samples were then subdivided into five aliquots of equal volume, and the viruses and nor-viruses were pelleted. Goat anti-TWEEN-ether disrupted R-MuLV serum, diluted 1:100 was then admixed with one pellet each of untreated R-MuLV and deglycosylated nor-R-MuLV. Each admixture was

maintained for one hour at 37°C to form antibody-virus or antibody-nor-virus adsorbates.

The virus and nor-virus were subsequently pelleted from each of the sera, and the respective sera were then sequentially adsorbed four additional times with the remaining viral pellets of each type, following the same general procedures. A portion of the serum was saved at each step so that at the end, antisera samples adsorbed 1x, 2x, 3x and 4x either with control (R-MuLV) or deglycosylated R-MuLV were obtained.

The sera were then assayed for neutralizing ability as described in detail hereinafter. The results are shown in FIGURE 4.

Those results indicate that deglycosylated, nor-R-MuLV adsorbed neutralizing antibodies as well as did untreated, native R-MuLV, since the neutralizing antibodies still recognized the deglycosylated virus. This was the case even though approximately 95 percent of the response by Western blotting was lost, as is shown in FIGURE 4.

Further assays were then conducted wherein rabbits were immunized by injection with inocula containing an effective amount of R-MuLV as control or deglycosylated, nor-R-MuLV in physiologically tolerable diluents to compare their immune responses, both in neutralization and by Western blotting techniques. As described before, the control sera preferentially reacted with untreated, rather than deglycosylated gp70, as shown in FIGURE 5. The antisera raised against deglycosylated, nor-R-MuLV, however, reacted equally well with both native and deglycosylated gp70 (p49) (FIGURE 5) and, overall, gave a much stronger antibody response.

These results illustrate that the carbohydrate moiety is not the only immunogenic part of gp70, since strong antibodies were raised in their absence. Additionally, the carbohydrate moieties masked immunogenic epitopes present in the protein backbone that were subsequently exposed upon deglycosylation.

In FIGURE 6, the results of neutralization assays are shown using sera from the before-mentioned rabbits. As can be seen in FIGURE 6, the nor-virus treated with Endo F elicited a much stronger initial neutralizing titer than did control virus when both virus and nor-virus were individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens. With multiple injections, however, the control virus-immunized rabbit eventually began producing more neutralizing antibodies, and its antibody-containing serum approached the titer of the Endo F-treated immunogen after five or six boosts. These results show that removal of carbohydrate enhances exposure of neutralizing sites, rather than abrogating the ability of the nor-virus to induce neutralizing antibodies.

These assays were repeated with FeLV. In that study, the deglycosylated nor-FeLV produced about twice the response in initial injections as control (undeglycosylated) FeLV, as shown in FIGURE 7. The results shown in FIGURE 7 again illustrate viral neutralization at a greater serum dilution by antibody-containing serum induced by a polypeptide of this invention (nor-virus) than was obtained by serum induced by the viral envelope N-linked glycoprotein (native virus) when the

polypeptide and viral envelope N-linked glycoprotein were individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

With respect to X47 influenza virus hemagglutinin, two rabbits have been injected with native X47 and deglycosylated X47, respectively, as described in detail hereinafter in Section III. Sera were collected from the rabbits. Neutralization assays have not yet been completed.

The above results illustrate that immunogenicity of a viral envelope N-linked glycoprotein can be improved according to the present invention by reacting the glycoprotein with an endoglycosidase, Endo F, that removes the asparagine, N-linked carbohydrate moieties (glycosyl groups) from the envelope glycoprotein to form the polypeptides and nor-viruses of the invention.

As the above results further illustrate, the polypeptides and nor-viruses of the invention are capable of inducing the production of antibody-containing serum that neutralizes the native virus at a greater serum dilution than does antibody-containing serum induced by the N-linked envelope glycoprotein or native virus, respectively, when the polypeptide or nor-virus and the N-linked envelope glycoprotein or virus, respectively, are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

C. Cross-Reactivity Studies

The seriological classification and subdivision of antigenically related viruses is known to result from the polymorphism of certain viral

proteins. Viral polymorphism reflects the response of specific viral genes to selective pressure and influences the dynamics of the host-parasite relationship. Such is the case for the hemagglutinin molecule of the influenza viruses, Fenner et al., "The Biology of Animal Viruses", 2d ed., Academic Press, New York (1973).

Another molecule whose polymorphism dictates host range, interference, and neutralization properties of the virus is the envelope glycoprotein gp70 of the murine and feline leukemia viruses (retroviruses). Friend, Maloney and Rauscher murine leukemia viruses (type C viruses) known as the FMR group have antigenically related gp70 glycoproteins.

For example, Niman and Elder, in "Monoclonal Antibodies and T-Cell Products", Katz ed., CRC Press, Boca Raton, Florida, pp 23-51 (1982), demonstrated FMR group gp70 antigenic cross-reactivities by generating monoclonal antibodies to Rauscher gp70 that immunoreact with the envelope gp70 glycoproteins of all FMR viruses. It is believed that the antigenic relatedness is the result of homologous amino acid residue sequences forming antigenic determinants common to the FMR group gp70 glycoproteins. The envelope glycoproteins of FeLV, HTLV I, HTLV II, HTLV III, MCF and ATLV are also believed to exhibit several homologous amino acid residue sequences among themselves as well as with the FMR group envelope glycoproteins.

Antigenic cross-reactivity based upon amino acid residue sequence homology is a phenomenon well known in the art. However, sequence homology does not guarantee cross-reactivity because the presentation of antigenic determinants may differ from one viral strain to another. For example,

R-MuLV and FeLV-B are believed to have gp70 sequence homology because of the known sequence homology between Friend murine leukemia virus (F-MuLV) and FeLV-B and the close serological relationship between F-MuLV and R-MuLV as demonstrated by Niman and Elder, supra. Yet, heteroserum made to native FeLV-B virion does not cross-react with R-MuLV gp70. This result, shown in FIGURE 8, suggests that antigenic determinants shared by R-MuLV and FeLV-B are not presented to their hosts in the same way.

The presentation of antigenic determinants in the R-MuLV gp70 system was also studied by Niman and Elder, supra. They demonstrated differential immunoprecipitation of native and deglycosylated gp70 by several monoclonal antibodies induced by native Rauscher gp70. It is believed that the differential immunoreactivity observed was due to carbohydrate side chains masking (sterically hindering) antigenic determinants.

To examine the possibility that carbohydrate side chains were masking antigenic determinants common to R-MuLV and FeLV-B, deglycosylated FeLV-B was used to induce antibodies in rabbits as described in detail hereinafter in Section III. As illustrated in FIGURE 9, antisera induced by deglycosylated, nor-FeLV-B immunoreacted with both native FeLV-B and nor-FeLV-B, as well as with native R-MuLV and deglycosylated R-MuLV gp70 glycoproteins.

It is thus seen that antibody-containing serum raised by immunization with one nor-virus immunoreacted with not only the envelope glycoprotein and carbohydrate-free envelope protein (polypeptide) of the immunizing nor-virus (nor-FeLV-B), but also immunoreacted with the envelope glycoprotein and carbohydrate-free envelope protein (polypeptide) of a

related viral strain; i.e., R-MuLV. Those results illustrate the improved cross-reactivity exhibited by the antibody-containing sera of the preseent invention.

Inasmuch as antigenic relatedness has been shown among the so-called FMR group of leukemia viruses, sequence homologies are known to be present among FeLV, HTLV I, HTLV II, HTLV III, MCF, and ATLV envelope glycoproteins, and cross-reactivity between serum of a patient with HTLV-II and a recombinant DNA-produced protein encoded by the env gene of HTLV-I has been reported [Samuel et al., Science, 226, 1094 (1984)], the illustration of improved cross-reactivity shown by the above-mentioned results also illustrates an improved cross-reactivity of antibody-containing serum induced by a nor-virus or carbohydrate-free envelope polypeptide of this invention among the nine specific, related viral strains named above, and among related leukemia viruses generally. Similar improvements in antibody-containing serum cross-reactivities also result among additional, related groups of virus strains whose native forms contain N-linked carbohydrate moieties on their envelope proteins when the methods disclosed herein are utilized. Exemplary of such additional, related groups of virus strains include strains of myxoviruses, measles viruses, rabies viruses and hepatitis B viruses.

D. Immunizations

The polypeptides or nor-viruses of this invention, when introduced into an animal host as a unit dose inoculum having an effective amount of polypeptide or nor-virus in a physiologically tolerable diluent, are capable of inducing production

of antibodies in the host mammal that immunoreact with the related virus, neutralize the virus in vitro, and preferably protect the host animal from in vivo infection caused by that virus.

The "effective amount" of polypeptide or nor-virus in a unit dose depends upon a number of factors. Included among those factors are the body weight of the animal immunized and the number of inoculations desired to be used. Individual unit dose inoculations typically contain about 10 micrograms to about 500 milligrams of polypeptide or nor-virus per kilogram body weight of the mammalian host. Inoculation methods and amounts in rabbits for the purpose of raising antibodies are described below.

Useful free polypeptides of this invention may be obtained from whole viruses by well-known techniques. See, for example, Niman and Elder, "Monoclonal Antibodies and T-Cell Products", supra; Niman and Elder, Virology, 123, 187 (1982); and Niman and Elder, Proc. Natl. Acad. Sci. (USA), 77, 4524 (1980). Influenza hemagglutinin may be obtained as described in Brown et al., J. Immunol., 125, 1583 (1980) and Aitken et al., Eur. J. Biochem., 107, 51 (1980). The resulting polypeptides are then deglycosylated with Endo F, as described in Section III hereinafter, and may then be utilized in the inocula of the invention.

Physiologically tolerable diluents are well known in the art, and alone are not part of the present invention. Exemplary of such diluents are distilled or deionized water, normal saline solutions and phosphate-buffered saline (PBS) solutions.

The immunizing composition or inoculum may be introduced into the host by intravenous injection, or the like, using known methods. Adjuvants such as

complete Freund's adjuvant (CFA), incomplete Freunds's adjuvant (IFA), alum, tetanus toxoid and the like as are well known in the immunological arts may also be included in the inocula as part of the physiologically tolerable diluent. Booster injections may also be given, as desired, to build a desired antibody titer in the host's serum.

Exact doses depend on the animal and polypeptide or nor-virus used, and can be determined using known challenge techniques. Additional exemplary amounts of immunogen such as the polypeptide or nor-virus, and specific reaction conditions for the inoculum preparation may be found in Bittle et al., Nature, 298, 30-33 (July, 1982).

The term "inoculum" is used herein to mean any immunizing composition. As such, the term also embraces vaccines that are useful in man and other mammals for conferring in vivo protection against a native virus. A given vaccine and inoculum may be identical where non-human mammalian hosts are involved, but typically differ where humans are the intended hosts. The reason for that difference is that adjuvants such as CFA are not utilized in humans, and another adjuvant must be used if any adjuvant is to be present in a human vaccine.

E. Antibodies

Antibodies to the polypeptides or nor-viruses of the present invention can be used in assays or to treat virus infections. The antibodies can be used directly as whole, intact antibodies or may be processed to provide Fab or $F(ab')_2$ portions, all of which are biologically active. The term "antibody" indicates a whole, intact antibody or the idiotype-containing polyamide portion of the antibody that is biochemically active and is capable

of immunoreacting with or binding to its antigenic ligand on the native virus, nor-virus or glycoprotein.

To manufacture antibodies, an immunizing inoculum described before is introduced into the host mammal as by injection. The host is maintained for a time sufficient for antibodies to be induced, usually for one to about four months. The desired antibodies raised are thereafter harvested from host fluids. The whole antibodies so induced can be used directly, or they may be cleaved with pepsin or papain as is well known to provide $F(ab')_2$ or Fab portions that may be used. The antibodies produced may also be used as therapeutic agents for passive immunoprophylaxis.

An animal infected by a retrovirus such as a leukemia virus or a myxovirus such as an influenza virus may be treated with antibodies preferably as whole antibodies raised to the polypeptides or nor-viruses of the present invention. The antibodies are administered in a unit dose having an effective amount of antibodies dispersed in a physiologically tolerable diluent such as saline or phosphate buffered saline.

An effective amount of such antibodies varies depending on the reactivity and type of the antibodies, but generally about 1 milligram to about 50 milligrams of antibody per kilogram animal weight is considered effective. In the case of mice and native murine leukemia virus, 1.5 milligrams of IgG antibody in ascites fluid were found to be effective in prolonging survival. See "Monoclonal Antibodies", Kennett et al. ed., Plenum Press (1980). The antibodies may be introduced intravenously or intraperitoneally, with several administrations given

at three to seven day intervals. The antibodies may also be given in conjunction with surgical treatment.

The antibodies may be obtained from sera of a second animal, different from the first animal to be treated, by raising antibodies to the polypeptides or nor-viruses of this invention. The antibodies may also be obtained from monoclonal sources such as ascites fluid by preparing a hybridoma cell line using known techniques. Whole antibodies are preferred as the antibodies since they are capable of activating the complement system when an immune complex is formed.

The term "unit dose" refers to physically discrete units suitable as unitary dosages for animals, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent or vehicle. The specifications for a novel unit dose of this invention are dictated by and are directly dependent on (a) the unique characteristics of the immunogen and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such active material for therapeutic use in animals.

III. Materials and Methods

A. Deglycosylation

Feline leukemia virus B (FeLV-B) and Rauscher murine leukemia virus (R-MuLV) obtained from the Resources Branch of the National Cancer Institute, Bethesda, MD were deglycosylated using endo-beta-N-Acetylglucosaminidase F (Endo F; Elder and Alexander, supra), that removes substantially all N-linked carbohydrate sidechains. A similar preparation of Endo F is also available from New England Nuclear (Boston, MA).

The removal of carbohydrate sidechains from R-MuLV was performed under three different virus denaturing (disrupting) conditions. Non-denaturing carbohydrate removal was accomplished by deglycosylating R-MuLV in deglycosylation buffer alone 10 millimolar (mM) sodium phosphate (pH 6.1), 5 mM EDTA and 0.15 molar (M) NaCl). Deglycosylation resulting in mild disruption of the nor-virus was performed using deglycosylation buffer containing 1 percent Nonidet P40. Carbohydrate removal was also performed under disrupting-reducing conditions using deglycosylation buffer containing 1 percent Nonidet P40 and 1 percent 2-mercaptoethanol. FeLV-B was deglycosylated using only the latter of the above buffer conditions.

Virus deglycosylated using non-denaturing conditions was first inactivated by exposing 2 milliliters (ml) of virus [0.1 milligram (mg)/ml in deglycosylation buffer] in an uncovered petri dish (Falcon model 1007; Falcon, Oxnard, CA) to ultraviolet light (uv) (Sylvania Germicidal model G30T8, GTE-Sylvania, Stamford, CT) for 5 minutes. Complete inactivation was confirmed by the inability to induce virus producing foci when used as a control in the appropriate neutralization assay described hereinafter.

The uv inactivated virus was then split into two 1 ml aliquots, one of which was incubated with 50 units of Endo F at 37°C for 16 hours, the control aliquot being similarly incubated without Endo F.

Deglycosylation under 1) mild disrupting and 2) disrupting-reducing conditions was performed by incubating 10 units of Endo F with 200 micrograms of virus in 200 microliters of the appropriate deglycosylation buffer for 16 hours at 37°C. Control

virus was similarly prepared without treatment with Endo F.

With respect to the X47 influenza virus, deglycosylation was accomplished by admixing $2x10^8$ plaque-forming units (pfu) of X47 influenza virus in 0.1M Tris HCl (pH 7.6), 0.1 percent sodium dodecyl sulfate (SDS), 1 percent 2-mercaptoethanol, and 0.05M EDTA. The resulting admixture was then immersed in boiling water for 2 minutes.

Subsequently, Nonidet P40 was added to provide a concentration of 1 percent. The admixture was cooled to 22°C and 20 units of Endo F were added thereto. The new admixture was then incubated at 22°C for 16 hours, immersed in boiling water for 2 minutes, and then cooled to 22°C. Then, an additional 20 units of Endo F were admixed therein and the resulting admixture was incubated at 22°C for 16 hours. At this point, the deglycosylated influenza virus was suitable for injection into rabbits.

B. Virus Neutralizing Antibody Adsorption

The ability of an antigen to immunoreact with antibodies indicates that the antigen contains determinants that are the same or similar to those immunogenic determinants that induced the antibodies. Therefore, the ability of virus-neutralizing antibodies induced by native virus to immunoreact with deglycosylated virus indicates the presence of virus-neutralizing immunogenic determinants on the deglycosylated virus.

The present assays used a virus-neutralizing antibody adsorption technique to examine whether deglycosylated virus (R-MuLV or FeLV-B) could adsorb out (immunoreact with) the neutralizing antibodies in

heterosera induced by native virus (R-MuLV or FeLV-B).

Goat anti-TWEEN-ether disrupted virion (R-MuLV or FeLV-B) was serially adsorbed (immunoreacted) four times using 200 micrograms of deglycosylated (uv inactivated, non-denatured) or control virus per adsorption. Illustratively, 1.5 ml of goat anti-gp70 antiserum diluted 1:100 using Minimum Eagle's Medium (MEM) containing 10 percent fetal calf serum was admixed (immunoreacted) with virus for 1 hour at 20°C with agitation. The reaction mixture was centrifuged for 5 minutes in a Fisher model 235A microcentrifuge to pellet the immunoreaction products.

A 200 microliter aliquot was removed from the supernantant and designated the 1x adsorption. The remaining supernatant was adsorbed against fresh virus following the above procedure. Further adsorptions were sequentially repeated until antiserum samples adsorbed 1x, 2x, 3x and 4x were obtained. These serially adsorbed serum samples were then assayed for their ability to neutralize native virus in the neutraliztion assay described hereinbelow.

C. Neutralization Assay

The immunoblotting neutralization assay described in Elder et al., Bio Techniques, 170-172, May-June, 1984 was used to examine, 1) the ability of deglycosylated virus to adsorb out neutralizing antibodies, and 2) the ability of antiserum induced by deglycosylated virus to neutralize native virus. The assay measured in vitro viral neutralization (inactivation) by immunoreaction between antibodies and live (infectious) virus particles. The assay was

reproducible and yielded a percent reduction in virus producing foci between control and experimental sera.

The type of cell line subjected to infection in the assay depended upon the virus under investigation because most viruses have limited host specificity. In the present assays, when the feline virus FeLV-B was being examined, the dog thymus cell line CF2th (ATCC CRL 1430, American Type Culture Collection, Bethesda, MD) was used as viral host. When the murine virus R-MuLV was being investigated, the mouse embryo cell line SC-1 (ATCC CRL 1404, American Type Culture Collection, Bethesda, MD) was used as viral host.

Both cell lines were grown in Minimum Eagle's Medium (MEM) supplemented with 10 percent fetal calf serum, penicillin (100 units/milliliter), streptomycin (100 milligrams/milliliter), 4 mM L-glutamine, and 1.0 mM sodium pyruvate. Following trypsinization with 0.025 percent trypsin in phosphate-buffered saline (PBS) and washing in MEM, $3X10^5$ cells were added to tissue culture petri dishes (Falcon #3003, Falcon, Oxnard, CA) and were incubated overnight at 37°C in a humidified atmosphere of 5 percent CO_2.

When the neutralization assay was used to examine the ability of deglycosylated virus to adsorb out neutralizing antibodies, native (untreated and infectious) virus (FeLV-B or R-MuLV) was immunoreacted with sera previously subjected to the virus neutralizing antibody adsorption technique described hereinabove. Non-adsorbed and normal rabbit sera were used as controls in addition to the control sera obtained during the adsorption.

When the neutralization assay was used to examine the ability of antiserum induced by

deglycosylated virus to neutralize native virus, native (untreated and infectious) virus (FeLV-B or R-MuLV) was immunoreacted with sera induced by nor-virus whose carbohydrate sidechains had been removed. Heteroserum raised against native virus and normal rabbit serum were used as controls.

Study and control immunoreactions were performed in separate containers by admixing 5 microliters of antiserum with 100 microliters of MEM containing 400 focus-forming units of virus. Each mixture was incubated at 37°C in a humidified 5 percent CO_2 atmosphere for 40 minutes. A solution of 5 milliliters of MEM containing 10 micrograms per milliliter hexadimethrine bromide (Polybrene P415, Sigma Chemical Co. St. Louis, MO) was then added. The resulting admixture was then used to inoculate the appropriate host cell culture.

The host cell cultures were inoculated by replacing the overnight growth MEM with 5 milliliters of the above admixture. This mixture was replaced after 24 hours and the cultures were incubated in MEM for an additional 4 day period at 37°C in a 5 percent CO_2 atmosphere. The cultures were then terminated by aspirating off the MEM, washing 3 times with 10 milliliters of PBS and allowing the monolayers to dry.

Virus-producing cell foci in the monolayer were detected by two techniques: (1) a radioimmune assay (RIA) and (2) an ELISA. Both assays were performed by pre-wetting a nitrocellulose disc designated BA85 (Schleicher & Schuell, Inc., Keene, NH) in PBS, placing the wetted disc over the monolayer and pressing firmly so as to transfer the cells from the petri dish onto the disc.

The cells were fixed on the disc by placing the disc in Amido Black Dye [0.045 percent Naphthol Blue Black (Sigma Chemical Co. St. Louis, MO), 45

percent methanol, 10 percent acetic acid] for 1 minute and then destained (45 percent methanol, 10 percent acetic acid) for 5 minutes. Non-specific binding sites were then blocked by incubating the discs in BLOTTO [Bovine Lacto Transfer Technique Optimizer, 5 percent weight per volume non-fat dry milk, 0.01 percent Antifoam A Emulsion (a 30 percent aqueous emulsion of a silicone polymer containing anionic emulsifiers, Sigma Chemical Co., St. Louis, MO), 0.0001 percent merthiolate (Sigma Chemical Co., St. Louis, MO) in PBS] overnight at 4°C, as in Johnson and Elder, J. Exp. Med., 159, 1751 (1983).

After blocking, each disc was placed in 5 milliliters of fresh BLOTTO with 50 microliters of goat anti-whole virus antiserum, (either anti-FeLV-B or anti-R-MuLV as appropriate) for 3 hours at 20°C while shaking. The discs were then washed with 5 milliliters of BLOTTO three times for 15 minutes each. The washed discs were placed in 5 milliliters of BLOTTO containing 5 microliters of rabbit anti-goat antisera and were incubated at 20°C for 1 hour with shaking. They were then again washed 3 times with BLOTTO.

Goat anti-whole virus antiserum was prepared by injecting either FeLV or R-MuLV virus particles into goats and recovering the sera. The rabbit anti-goat antisera were prepared by injecting rabbits as described above using purified goat gamma-globulin as immogen in a physiologically tolerable diluent.

For the RIA, Staphylococcus aureus Protein A was labeled with ^{125}I, and 5 milliliters of BLOTTO containing 2.5 microliters of ^{125}I-labeled Protein A was incubated with the discs for 1 hour at 20°C with shaking. This allowed the labeled Protein A to bind with the bound rabbit anti-goat antibodies. The

discs were again washed with lithium chloride buffer [0.5 M LiCl (Fisher Scientic, Pittsburgh, PA), 0.1 M Tris Base (Sigma Chemical Co. St. Louis, MO] for 30 minutes and finally with water for 30 minutes. The washed discs were dried and exposed to Kodak XRP-1 x-ray film available from Eastman Kodak Company, Rochester, NY.

For the ELISA, peroxidase-coupled goat anti-rabbit IgG (Tago, Burlingame CA) was diluted 1:500 in BLOTTO. The BLOTTO-washed discs were then immersed in an amount of the diluted, peroxidase-coupled goat anti-rabbit IgG composition to cover the discs, and maintained for a time period of 1.5 hours to bind the peroxidase-labeled goat antibodies to the disc-bound rabbit antibodies. The discs were thereafter washed in a bath of BLOTTO for 10 minutes followed by a washing in a deionized water bath for 10 minutes.

An ELISA-developing bath was prepared by admixing the following: 8 ml of a solution of 4-chloro-2-naphthol (3 mg/ml in methanol), 4 ml of 1 molar sodium acetate (pH 5.9), 41 ml of water and 30 microliters of 30% hydrogen peroxide. The above, washed discs were immersed in a sufficient amount of developing bath to cover the discs, and maintained in the bath for a period of 2-5 minutes. The color development reaction was stopped by washing the discs with water, and the discs were air dried.

The Western blot analyses of FIGURES 5, 8 and 9 were also visualized following the above ELISA technique.

Results of the assays are shown the graphs of FIGURES 4, 6 and 7. The percent neutralization was calculatd from the number of foci on any given

disc treated with an antiserum compared to the number of foci on a control disc treated with control sera.

D. Western Blotting

The Western Blot technique was done according to published procedures and was used to examine the ability of heteroserum raised against the whole virus to bind deglycosylated gp70. The viral proteins were separated by gradient (5-17.5 percent) SDS-polyacrylamide gel electrophoresis. See Laemmli, Nature, supra, and Towbin, et al., Proc. Natl. Acad Sci. (USA), supra.

Proteins were electrophoretically transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) as described by Towbin et al., Proc. Natl. Acad. Sci. (USA), 76, 4350 (1976), using an electroblot apparatus, (E.C. Apparatus Corp., Jacksonville, FL) with buffer consisting of 25 millimolar Tris Base, 192 millimolar glycine, 20 percent methanol and 0.1 percent sodium dodecyl sulfate (pH 8.3). Following the transfer, the nitrocellulose was blocked in BLOTTO to reduce non-specific binding. The resulting blots were reacted with 100 microliters of heteroserum to R-MuLV or FeLV as appropriate in 10 milliliters of BLOTTO for 3 hours, and were then washed three times for 1 hour with 50 milliliters of fresh BLOTTO.

Antibodies bound to specific control and deglycosylated viral proteins were detected by one of two methods. In the first method, antibodies were detected by reacting the blots with 20 microliters of ^{125}I-labeled Protein A in 10 milliliters of BLOTTO for 1 hour. The blots were then washed four times in 50 milliliters of fresh BLOTTO for 15 minutes and then under a continuous flow of water for 20 minutes.

In the second method, such antibodies were detected by reacting the blots with peroxidase-coupled goat anti-rabbit IgG, hydrogen peroxide and o-dianisidine as described in FIGURE 3.

E. Immunizations

The antisera against deglycosylated, nor-virus used in the neutralization assays described herein were obtained from immunized rabbits. Control sera were obtained from rabbits by bleeding just prior to initial immunization.

Antiserum to deglycosylated nor-R-MuLV was obtained by immunization with 200 micrograms nor-R-MuLV, deglycosylated under mild disrupting conditions, as described hereinabove, in complete Freund's adjuvant (CFA), in incomplete Freund's adjuvant (IFA) and on alum (5 milligrams/milliliter) on days 0, 14 and 21, respectively. Thereafter, the rabbit was boosted with nor-R-MuLV, deglycosylated under disrupting-reducing conditions, as described hereinabove, or alum on a monthly basis. Rabbits immunized with influenza-related immunogens were injected with inocula containing $2X10^7$ plaque-forming units of virus or nor-virus following the above immunization regimen.

Each immunization consisted of introducing the inoculum in four subcutaneous injections of 50 micrograms of deglycosylated, nor-virus, one on each shoulder and hip. Rabbits were bled 7 days after the last immunization, and in some cases, the rabbits were further boosted, as described above, with immunogen in alum and bled as necessary. The collected blood was allowed to clot overnight and then was centrifuged to obtain the sera used in the assays discussed hereinabove.

In addition to the adjuvants, the inocula also contained a physiologically tolerable diluent such as water, or phosphate-buffered saline (pH 7.4). Inocula stock solutions were prepared with CFA or IFA as follows: An amount of deglycosylated nor-virus conjugate sufficient to provide the desired amount of deglycosylated nor-virus inoculation was dissolved in phosphate-buffered saline (PBS). Equal volumes of CFA or IFA were then mixed with the deglycosylated nor-virus conjugate solution to provide an inoculum containing deglycosylated nor-virus, water and adjuvant solution in which the water to oil ratio was 1:1. The mixture was thereafter homogenized to provide the inoculum stock solution.

The foregoing is intended as illustrative of the present invention but not limiting. Numerous variations and modifications may be effected without departing from the true spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A polypeptide comprising an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, said polypeptide being substantially free of the N-linked glycosylation of said glycoprotein and inducing the production of antibody-containing serum that neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by the viral envelope N-linked glycoprotein when the polypeptide and the viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

2. The polypeptide of claim 1 wherein said glycoprotein is a glycoprotein of feline leukemia virus-B.

3. The polypeptide of claim 1 wherein said glycoprotein is surface glycoprotein gp70 of Rauscher murine leukemia virus.

4. The polypeptide of claim 1 wherein said antibodies induced by said polypeptide provide improved cross-reactivity among related viral strains.

5. A nor-virus whose envelope protein has an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, said envelope protein of said nor-virus being substantially free of carbohydrate moieties covalently linked to asparagine residues and inducing the production of antibody-containing serum that neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing

serum induced by said virus, when said nor-virus and said virus are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

6. The nor-virus of claim 5 wherein said glycoprotein is a glycoprotein of feline leukemia virus-B.

7. The nor-virus of claim 5 wherein said glycoprotein is surface glycoprotein gp70 of Rauscher murine leukemia virus.

8. The nor-virus of claim 5 wherein said glycoprotein is X47 influenza virus hemagglutinin.

9. A nor-virus whose envelope protein has an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, said envelope protein of said nor-virus being substantially free of carbohydrate moieties covalently linked to asparagine residues and inducing the production of antibody-containing serum that provides improved cross-reactivity among related viral strains, when said nor-virus and the virus of said viral glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

10. The nor-virus of claim 9 wherein said glycoprotein is surface glycoprotein gp70 of Rauscher murine leukemia virus.

11. The nor-virus of claim 9 wherein said glycoprotein is X47 influenza virus hemagglutinin.

12. The nor-virus of claim 9 wherein said glycoprotein is a glycoprotien of feline leukemia virus-B.

13. The nor-virus of claim 9 wherein said virus is a retrovirus.

14. The nor-virus of claim 9 wherein said virus is a myxovirus.

15. The nor-virus of claim 9 wherein said antibodies induced by said nor-virus neutralize said virus at a greater serum dilution than do said antibodies induced by said virus.

16. An inoculum against infection by a retrovirus comprising an effective amount of (1) a polypeptide comprising an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, said polypeptide being substantially free of the glycosylation of said N-linked glycoprotein and inducing the production of antibody-containing serum that (i) provides improved cross-reactivity among related viral strains and (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by said viral envelope N-linked glycoprotein when said polypeptide and said viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens, and (2) a physiologically tolerable diluent, said inoculum, when introduced into a host, being capable of inducing the production of antibodies in the host that immunoreact with said retrovirus, neutralize said retrovirus in vitro, and protect the host from in vivo retroviral infection.

17. The inoculum of claim 16 wherein said glycoprotein is a surface glycoprotein of said retrovirus.

18. An inoculum against infection by a myxovirus having an effective amount of (1) a polypeptide comprising an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, said polypeptide being substantially free of the N-linked glycosylation of said glycoprotein and inducing the production of antibody-containing serum that (i) provides improved cross-reactivity among related viral stains and (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by said viral envelope N-linked glycoprotein when said polypeptide and said viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens, and (2) a physiologically tolerable diluent, said inoculum, when introduced into a host, being capable of inducing the production of antibodies in the host that immunoreact with said myxovirus, neutralize said myxovirus in vitro, and protect the host from in vivo myxoviral infection.

19. The inoculum of claim 18 wherein said glycoprotein is an influenza virus hemagglutinin.

20. Antibodies to retrovirus, said antibodies being raised in an animal host to a polypeptide, said polypeptide comprising an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, said polypeptide being substantially free of the N-linked glycosylation of said glycoprotein and inducing the production of antibody-containing serum that (i) provides improved cross-reactivity among related viral strains and (ii) neutralizes the virus of said

viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by said viral envelope N-linked glycoprotein when said polypeptide and said viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens, said antibodies having the capacity to immunoreact with said retrovirus and neutralize said retrovirus in vitro.

21. The antibodies of claim 20 wherein said glycoprotein is a surface glycoprotein of said retrovirus.

22. The antibodies of claim 20 wherein said retrovirus is Rauscher murine leukemia virus.

23. The antibodies of claim 20 wherein said retrovirus is feline leukemia virus-B.

24. Antibodies to myxovirus, said antibodies being raised in an animal host to a polypeptide, said polypeptide comprising an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, said polypeptide being substantially free of the N-linked glycosylation of said glycoprotein and inducing the production of antibody-containing serum that (i) provides improved cross-reactivity among related viral strains and (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by said viral envelope N-linked glycoprotein when said polypeptide and said viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens, said

antibodies having the capacity to immunoreact with said myxovirus and neutralize said myxovirus in vitro.

25. The antibodies of claim 24 wherein said glycoprotein is an influenza virus hemagglutinin.

26. A method for the production of antibodies that neutralize a virus comprising the steps of:

(a) introducing into an animal host an effective amount of a polypeptide comprising an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, said polypeptide being substantially free of the N-linked glycosylation of said glycoprotein and inducing the production of antibody-containing serum that (i) provides improved cross-reactivity among related viral strains and (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by said viral envelope N-linked glycoprotein when said polypeptide and said viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens, said polypeptide being capable of inducing the production of antibodies in the serum of said host; and

(b) maintaining said host for a period of time sufficient for antibodies to be induced; and

(c) collecting said induced antibody-containing serum from said host;

said antibody-containing serum immunoreacting with said virus and neutralizing said virus in vitro.

27. The method of claim 26 wherein said virus is a retrovirus.

28. The method of claim 26 wherein said virus is a myxovirus.

29. A method of improving the immunogenicity of a viral envelope N-linked glycoprotein comprising the steps of:

(a) providing said viral envelope N-linked glycoprotein; and

(b) reacting said glycoprotein with a glycosidase, said glycosidase removing glycosyl groups from said glycoprotein to form a polypeptide,

said polypeptide being capable of inducing the production of antibody-containing serum that (i) provides improved cross-reactivity among related viral strains and (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by said viral envelope N-linked glycoprotein when said polypeptide and said viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

30. The method of claim 29 wherein said glycosyl cleavage occurs at the asparagine residue on said glycoprotein.

31. The method of claim 29 wherein said glycoprotein is surface glycoprotein gp70 of Rauscher murine leukemia virus.

32. The method of claim 29 wherein said glycoprotein is X47 influenza virus hemagglutinin.

33. The method of claim 29 wherein said glycoprotein is a glycoprotein of feline leukemia virus-B.

34. The method of claim 29 wherein said native virus is a retrovirus.

35. The method of claim 29 wherein said native virus is a myxovirus.

36. A method of immunizing an animal against a virus comprising the steps of:

(a) providing a unit dose of inoculum comprising a polypeptide of claim 1 dispersed in an effective amount in a physiologically tolerable diluent, said polypeptide having the capacity to induce the production of antibodies in said animal that immunoreact with said virus, neutralize said virus in vitro, and protect said animal from said virus; and

(b) injecting the unit dose of said inoculum into the blood stream of the animal to be immunized;and

(c) maintaining said host for a period of time sufficient for protective antibodies to be induced.

37. The method of claim 36 wherein said virus is a retrovirus.

38. The method of claim 36 wherein said virus is a myxovirus.

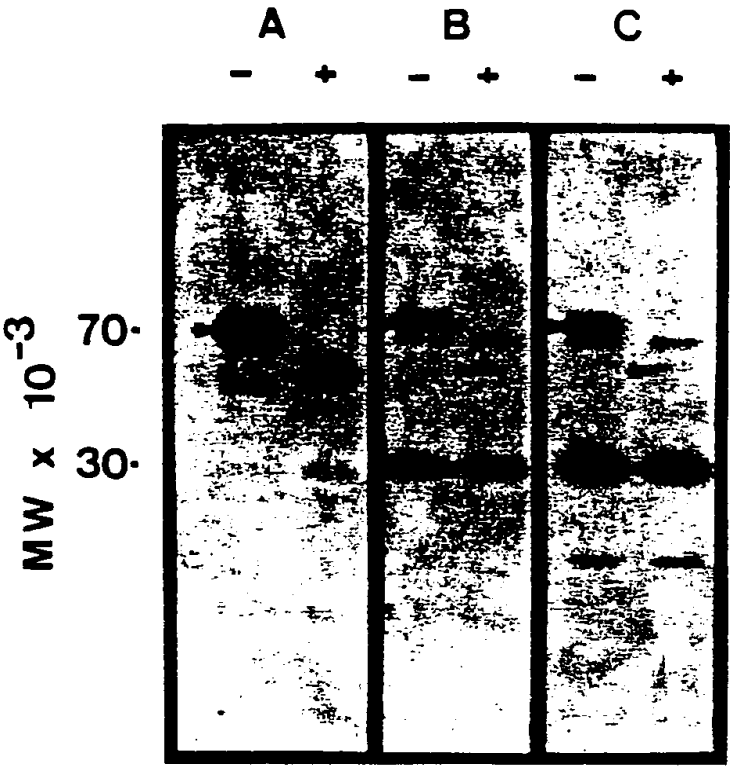
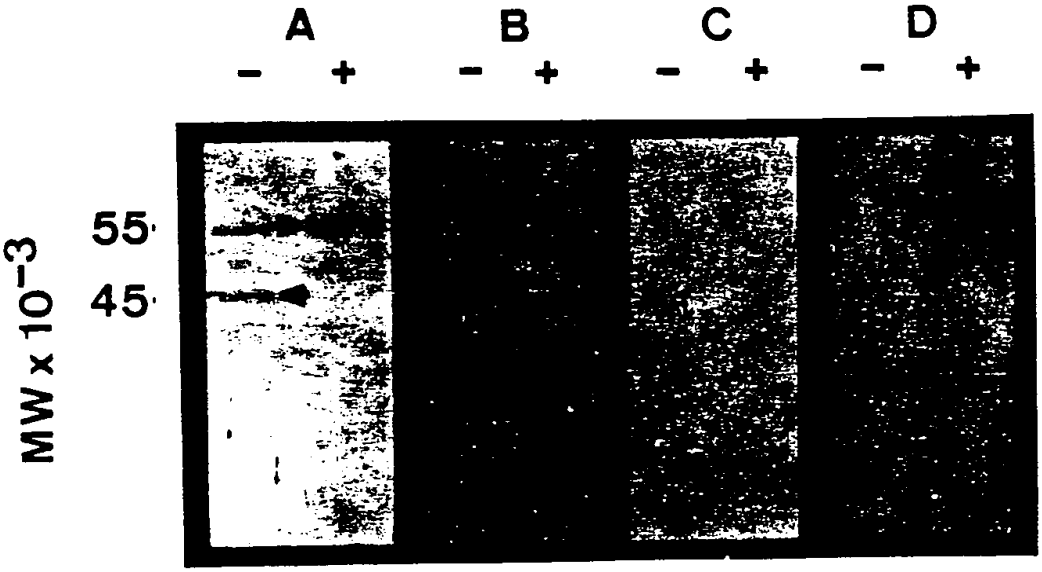


FIG. 1

FIG. 3



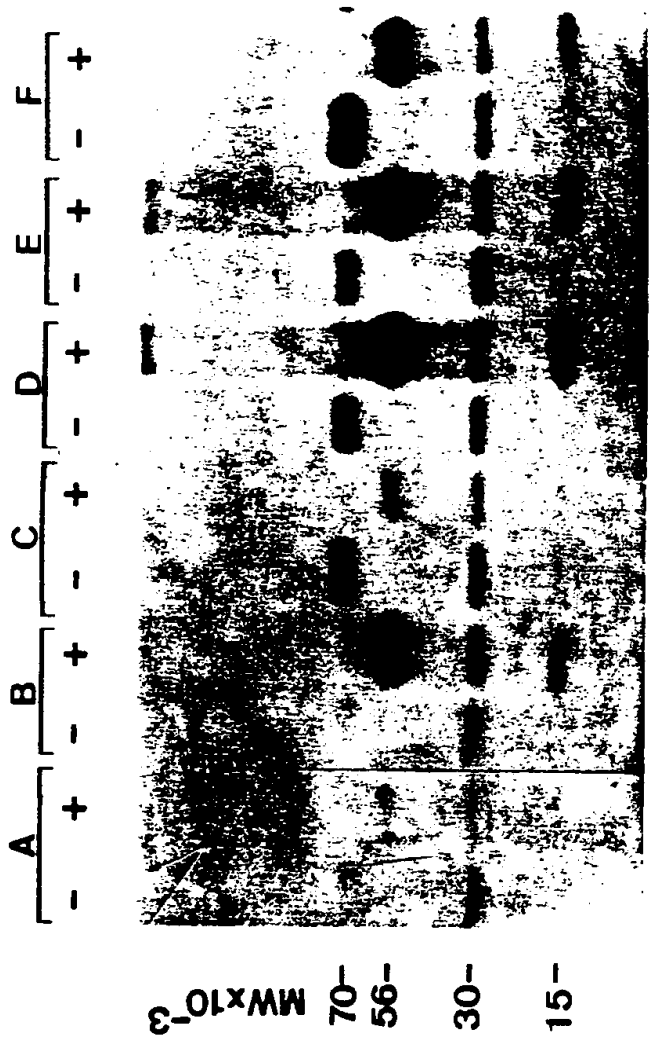


FIG. 2

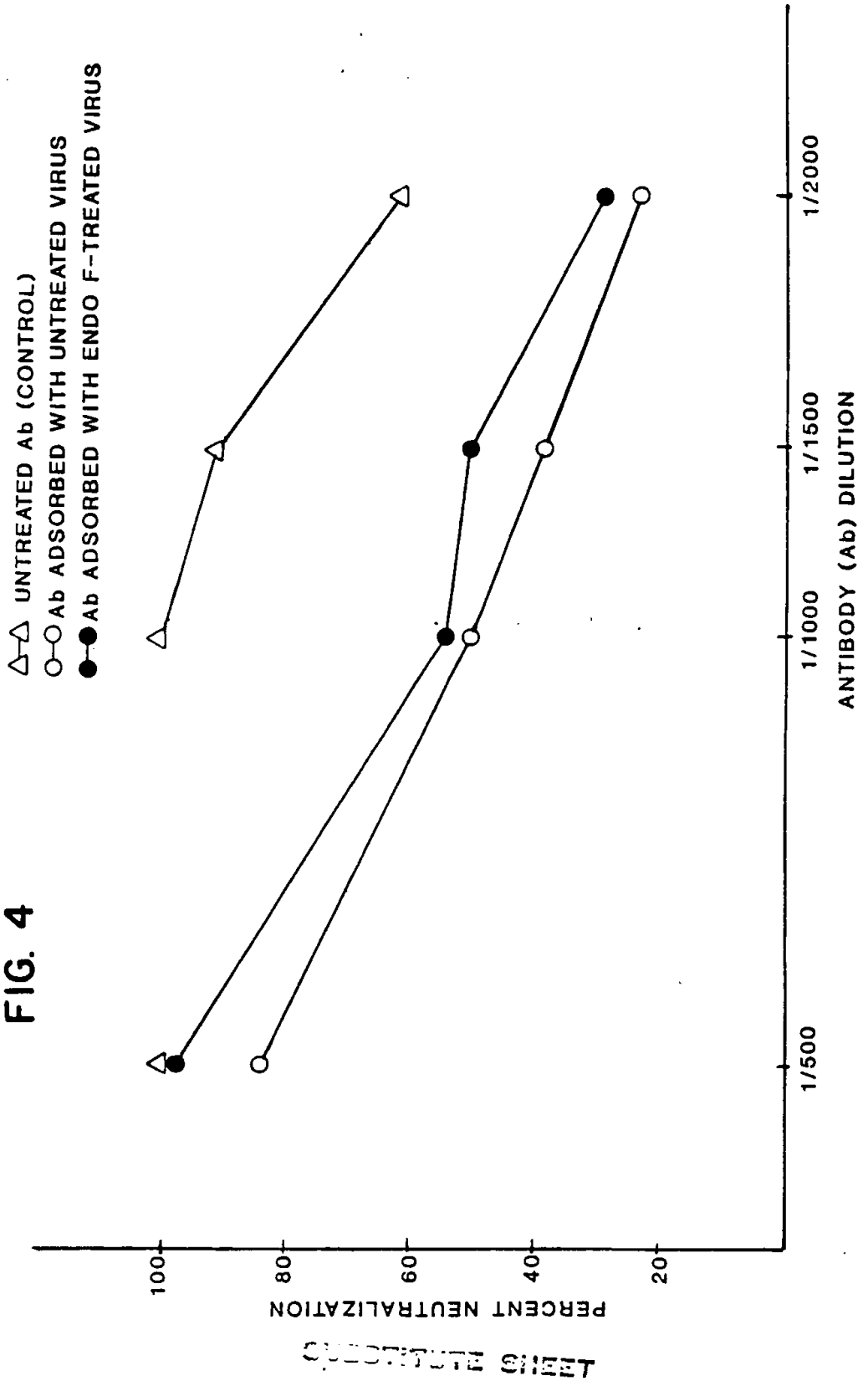


FIG. 4

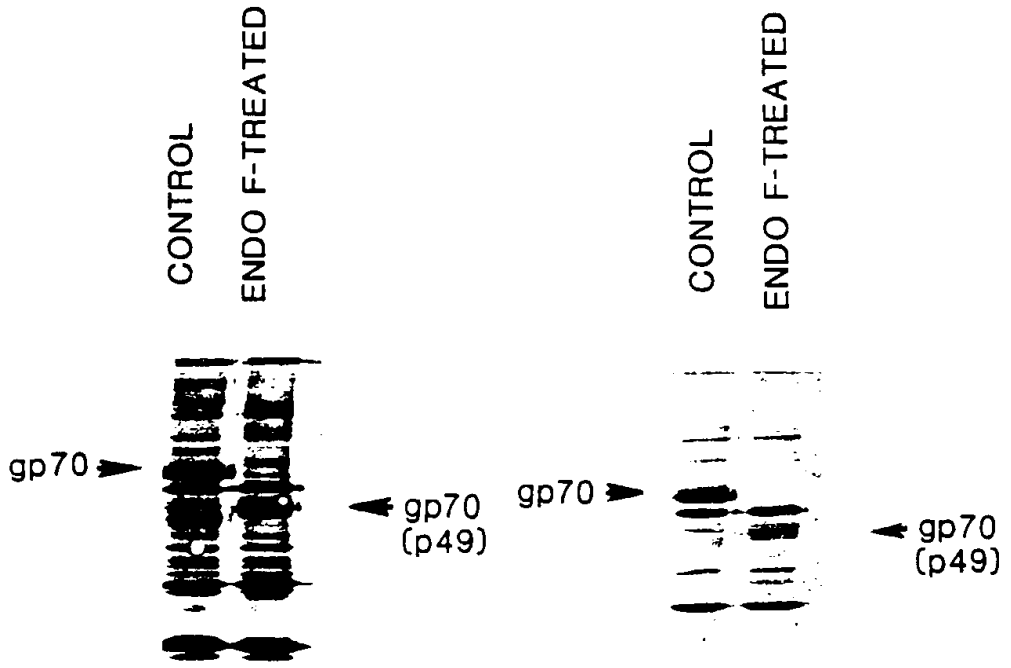

CONTROL
ENDO F-TREATED
gp70
gp70
(p49)
CONTROL
ENDO F-TREATED
gp70
gp70
(p49)

FIG. 5

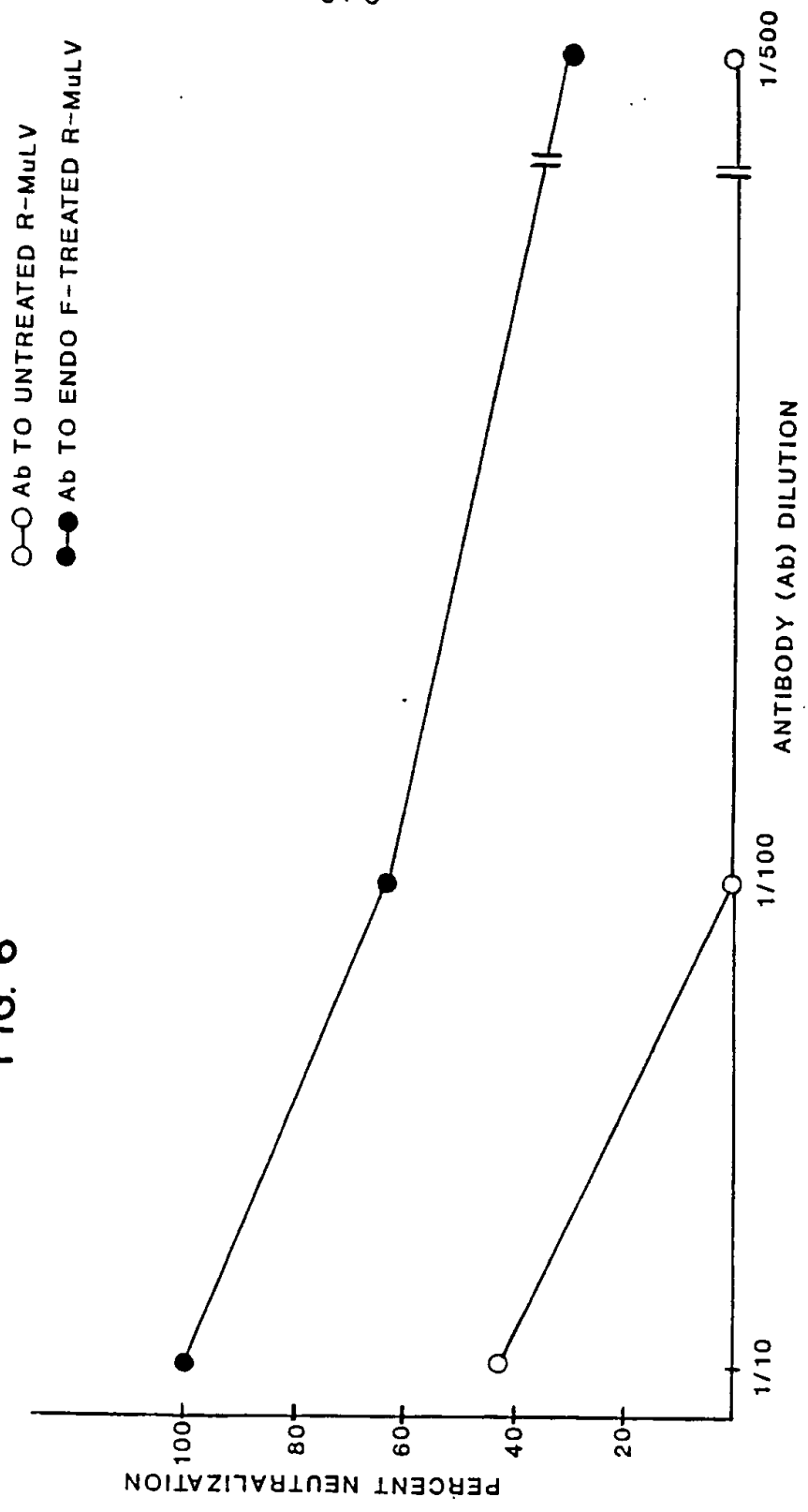

Ab TO UNTREATED R-MuLV
Ab TO ENDO F-TREATED R-MuLV
PERCENT NEUTRALIZATION
100
80
60
40
20
1/10
1/100
1/500
ANTIBODY (Ab) DILUTION

FIG. 6

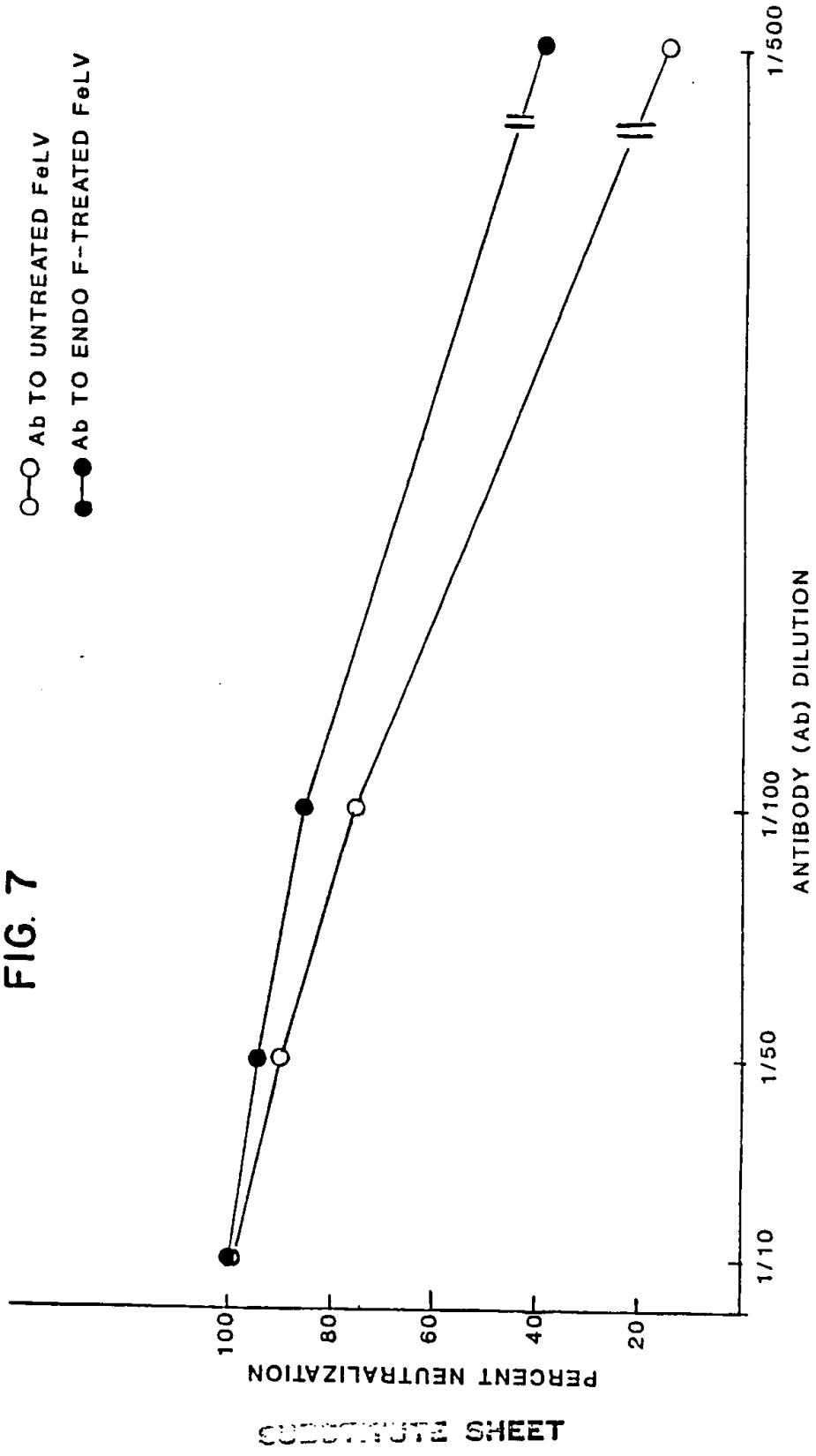

Ab TO UNTREATED FeLV
Ab TO ENDO F-TREATED FeLV
PERCENT NEUTRALIZATION
100
80
60
40
20
ANTIBODY (Ab) DILUTION
1/10
1/50
1/100
1/500

FIG. 7

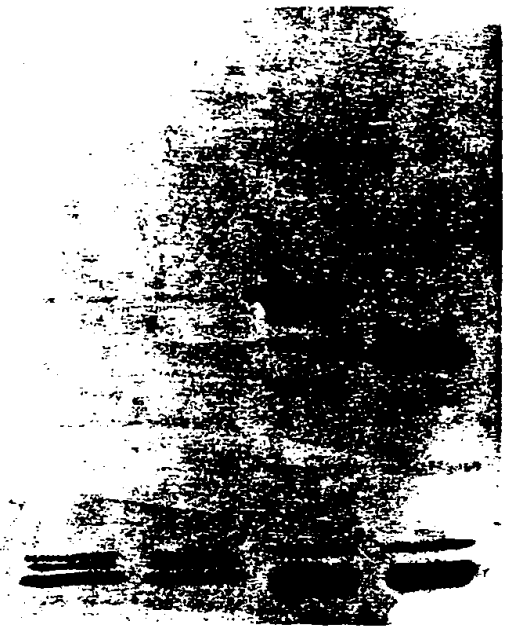


FIG. 8

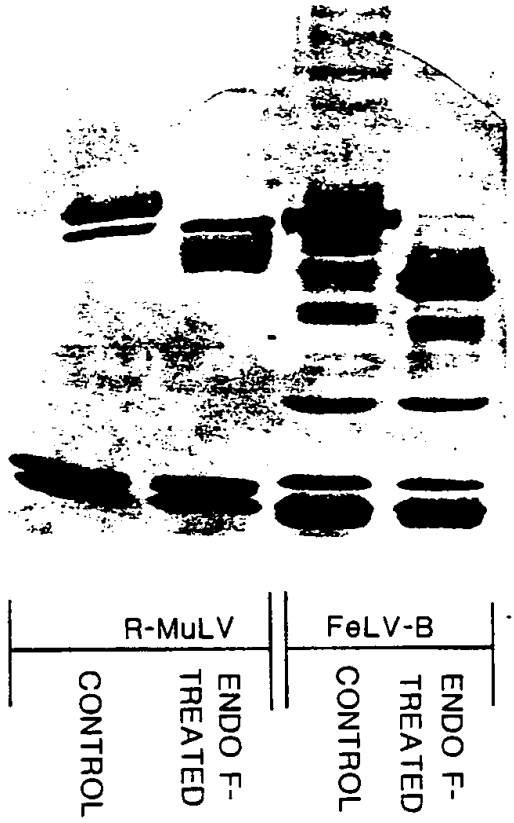


FIG. 9

INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/02319

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S. CL. 435/235 INT. CL.[4] C12N 7/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/68,235,236,238 424/89,93

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [6]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [5]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A	Proc. Natl. Acad. Sci. USA Vol. 77, p.1622-1626. 1980. H. Snyder and E. Fleissner. Specificity of human antibodies to oncovirus glycoproteins: Recognition of antigen by natural antibodies directed against carbohydrate structures.	1-38
Y	Virology, Vol. 109, p.431-434. 1981. M. Schmerr et al. Antigenic Reactivity of a soluble glycoprotein associated with bovine leukemia virus.	1-38
Y	Proc. Natl. Acad. Sci USA Vol.79, p.4540-4544. 1982. J.Elder and S. Alexander. endo-B-N-Acetylglucos-aminidase F: Endoglycosidase from Flavobacterium meaningosepticum that cleaves both high-mannose and complex glycoproteins.	1-38

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [1]	Date of Mailing of this International Search Report [2]
February 18, 1986	27 FEB 1986
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Michael Rabson

Form PCT/ISA/210 (second sheet) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where apropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	Nature Vol. 298, p.30-33. 1982. J. Bittle et al. Protection against foot-and-mouth disease by Immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence.	16-38
Y	Virology Vol. 136, p.20-31. 1984. C. Bruck et al. Biologically active epitopes of bovine leukemia virus glycoprotein GP 51.	1-15
P,X	Science Vol. 226, p.1328-1330. Dec. 1984. S.Alexander and J. Elder. Carbohydrate dramatically influences immune reactivity of antisera to viral glycoproteins antigens.	1-38